IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Serial No.: 10/790,992
Filed: 03/02/2004
Applicant: Nemenov
For: Portable Laser and Process for Producing Controlled Pain

Declaration under 37 CFR Section 1.131
Proving Invention Prior to Collaboration with Co-Authors

Applicant Mikhail Nemenov declares as follows:

1. I make this declaration in support of my claim that the invention currently claimed according to the amendment filed today in the subject patent application was conceived by me alone prior to my collaboration with the co-authors of the attached paper accepted 1 March 2002 and published in the journal Pain (herein referred to as the Greffrath paper and attached as Attachment 1). My co-authors on the paper were Drs. Wolfgang Greffrath, Stefan Schwarz, Ulf Baumgartner, Hagen Vogel, Lars Arendt-Nielsen and Rolf-Detlef Treede. The claims have been amended to cancel Claim 23 which was derived from my collaboration with Dr. David Clifford Yeomans. Dr Yeomans has consented to his being removed as a co-inventor and a request that he be removed is being filed with the amendment.
2. There has been for several years a need in nerve research to be able to perform experiments in which only a single nerve is stimulated. The potential for use of laser power for nerve stimulation has been known for many years. The separation of nerve endings in human skin tissue varies from about 0.5 mm in tissue such as fingertips to a few centimeters in regions of the back. In most regions of the skin the nerve endings are separated by about 1 to 3 mm. Therefore, in most skin regions, if the laser power is insufficient, too much time is required to provide enough energy to stimulate a single nerve and the laser energy dissipates affecting multiple nerves. YAG:Nd lasers have been available for many years that have sufficient power to stimulate single nerves with a properly focused beam.
3. During the period 1994-1995, I performed experiments with a YAG:Nd laser operating at 1,060 nm to evoke single cell (monomodal) sensations (including pain sensations). The results of these YAG:Nd lasers clearly demonstrated that single nerves could be stimulated with the pulsed laser beam from these YAG:Nd lasers. Results of these tests were not published. YAG:Nd lasers of the type I used were large (about 1 m X 0.3 m X 0.3 m) and heavy (about 100 kg) and expensive (about ($ 60,000). Diode lasers are at that time were easily portable and cost only about $ 10,000.
4. During the period 1996 – 1997 diode lasers with sufficient power to stimulate single nerves became available and I developed a 20 W diode laser device. Six volunteers were tested in facilities of my lab at Pavlov Medical University in Saint Petersburg, Russia during the period Dec 1996 to April 1997. With these test I was able to confirm in Russia my concept of producing single cell

stimulation with a small relatively low cost diode laser. This was my first actual reduction to practice of my concept of using diode lasers for single nerve stimulation. The results of these experiments were not published but the results were discussed privately at a workshop that I helped organize in May 1997 at Pavlov Medical University. A copy of workshop agenda is attached as Attachment 2. My oral presentation is referred to at Section 3.2.

5. At the May 1997 workshop I met Dr Arendt-Nielsen who operates a research laboratory in Denmark that is well-known for its nerve research. I proposed to him formal experiments to confirm my earlier results that the relatively inexpensive diode lasers could be used for monomodal skin experiments and Dr. Arendt-Nielsen faxed me a formal invitation to apply for work permit in Denmark.
6. During the period January – April 1998, I developed specifications, protocol and tested a prototype diode laser in preparation for formal nerve research on volunteers at Dr. Arendt-Nielsen's lab. This preliminary project was funded at my own my expense. During January 1998 I contacted IRE Polus, a laser company and requested specifications for various models of their diode lasers. A copy of response of laser company IRE-Polus to my request is attached as Attachment 3.
7. In May 1998 Dr. Arendt-Nielsen invited me to conduct experiments in his lab in Aalborg, Denmark. He agreed to pay for the parts for a new laser built to my specifications. The parts for the diode laser for Dr. Arendt-Nielsen lab were ordered in April of 1998 from IRE-Polus. The confirmation of order was done in the June of 1998. Attachment 4 is a copy of an invoice for the parts.
8. During the period July – December 1998 the diode laser for Dr. Arendt-Nielsen lab was fabricated, tested and applied to volunteers. I supervised a student of Aalborg University who developed interface software in accordance with protocols that I developed that simplified the stimulation set up. About 30 volunteers were tested at the Arendt-Nielsen laboratory but these experiments were cancelled before results good enough to be reported in scientific literature could be obtained.
9. In November-December of 1998. I contacted Dr. Treede who directs a research laboratory in Main University in Denmark and suggested that I would like to perform nerve stimulation studies in his laboratory. . A copy of my initial email and his response is attached as Attachment 5. I told him I thought I could prove that diode lasers could be used to produce single monomodal sensation in skin and activation of single nerve in vitro. We agreed that I would provide the laser and protocol. Dr. Treede asked when I could have a laser ready to use in the study. I told him that I could have a laser and protocol by the end of July 1999. The preparation of the laser and the protocol was funded at my own expense.
10. Dr. Treede arranged for a travel grant to support my travel and to cover lodging for the experiment. During September 16 – 28, 1999, I performed nerve experiments in Dr. Treede's laboratory with my diode laser. My work was a part of a team of researchers including Drs. Baumgartner, Vogel, Schwartz and Greffrath who were all employees or otherwise affiliated with Dr Treede's laboratory. Dr. Baumgartner was not present at the actual experiments but he processed EEG laser evoked potentials in human experiments. Dr. Vogel

supervised the recording of EEG/LEP data in accordance with a protocol I developed. The actual recording of the data was by a technician in the laboratory whose name I do not remember. The recording of the data was accomplished in one day. Dr. Schwartz prepared and assembled a calibrations patch for use in experiments. Dr. Greffrath prepared all nerve cells and tested the cells to determine if they were capsaicin sensitive. I applied the laser to the cells. These in vitro experiments took about 5 days because for statistically proven data we need about 6-8 capsaicin sensitive cells. Dr. Greffrath wrote most of the 2002 paper published in the journal Pain. A copy of the paper (as previously stated) is attached to this declaration as Attachment 1. The work was supported by a grant as explained in the last sentence of the Pain paper. My travel grant was from a Germany academic fund (DAAD grant) as a visiting professor. This was the second actual reduction to practice of my concept of using diode lasers to produce single nerve stimulation. During the period between my first actual reduction to practice (in December 1996 to April 1997) and the second September 1999 reduction to practice my efforts to prove that diode lasers could be used for single nerve stimulation were continuous and diligent.

11. In October 2000 I moved to the United States and was employed at Sparkolor Corp which at the time was developing diode lasers. I applied for Permanent Residency in the United States. In support of my application for a "Green Card" Dr. Treede wrote a letter setting forth facts relating to my earlier pain research using diode lasers. A copy of that letter is attached as Attachment 6. My effort to promote the use of diode lasers for single nerve stimulation has been continuous and uninterrupted since diode lasers first became powerful enough for this use in the 1996 period.
12. After the work at the Treede laboratory was completed, I continued to develop procedures for nerve stimulation with my diode laser. During the period from September 2002 to March 3, 2003 I collaborated with Dr David Clifford Yeomans. We conducted rat experiments similar to some of the experiments in the Treede laboratory. That collaboration resulted in improvements to the processes that I had first reduced to practice during the period Dec 1996 to April 1997 that had been utilized in work at the Arendt-Nielsen and Treede laboratories. Specifically Dr. Yeomans contributions led to the specific improvements relating laser pulse energy, timing and duration. These improvements were claimed in Claim 23. This claim has been cancelled and a request to remove Dr. Yeomans as a co-inventor is being filed along with the amendment and this declaration. This action is being done to simplify what is the claimed invention and to simplify the timing of the conception of the invention.
13. In February 2003 I contacted a San Diego patent attorney, John R. Ross who assisted me in preparing a provisional patent application on March 3, 2003 and on March 2, 2004 he filed on my behalf the present patent application.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U. S. C.

1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

X _____ 01/08/2007

Michael Nemcnov Date

Attachment 1
pp 145-155

PAIN

Pain 99 (2002) 145–155

www.elsevier.com/locate/pain

Inward currents in primary nociceptive neurons of the rat and pain sensations in humans elicited by infrared diode laser pulses

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Received 21 June 2001; received in revised form 22 February 2002; accepted 1 March 2002

Abstract

Radiant heat is often used to study nociception in vivo. We now used infrared radiation generated by a diode laser stimulator (wavelength 980 nm) to investigate transduction mechanisms for noxious heat stimuli in acutely dissociated dorsal root ganglion (DRG) neurons of rats in vitro. The laser stimulator offered the unique opportunity to test whether the same stimuli also elicit pain sensations in humans. A specific heat-induced current (I_{heat}) was elicited in six of 13 small DRG neurons (diameter ≤30 μm) tested in the whole-cell configuration of the patch–clamp mode. Current responses in the seven heat-insensitive neurons were within the range explainable by the temperature dependence of the recording setup. I_{heat} was characterized by: (1) non-linearity of its amplitude during a suprathreshold heat ramp as well as with stimuli of increasing intensity with an estimated threshold of 42 ± 1°C; (2) fast rise time and even faster decay time ($t_{1/2}$ = 96.5 ± 5.9 and 27.7 ± 1.5 ms, respectively); and (3) rate dependence of its induction. All three heat-sensitive neurons tested were also sensitive to capsaicin. The mean threshold for the induction of I_{heat} was 2.8 ± 0.3 J mm^{-2}. The threshold for the induction of action potentials by depolarizing current pulses was significantly reduced after laser stimulation, suggesting a sensitization at the transformation stage. No such change was seen in heat-insensitive neurons that underwent the same heat stimuli. The same diode laser elicited pain sensations and laser-evoked potentials in human subjects. No significant differences were seen between the pain thresholds in hairy and in glabrous skin, probably due to the deep penetration of this laser radiation. The mean pain threshold for stimuli ≥200 ms in humans was 2.5 ± 0.2 J mm^{-2} (n = 11), and did not differ from the thresholds for the induction of I_{heat} in vitro. Our results indicate that I_{heat} in primary sensory neurons can be activated by infrared laser pulses that are painful in humans.

Keywords: Noxious heat; Dorsal root ganglion; Evoked potential; Psychophysics

1. Introduction

Stimulation of the human skin with radiant heat stimuli generated by infrared lasers typically leads to a stinging and/or burning sensation. This painful sensation is mediated through activation of peripheral endings of Aδ- and C-fiber nociceptors (Bromm and Treede, 1984; Treede et al., 1995; Magerl et al., 1999). The neural discharges are integrated in the spinal cord and then carried via the spinothalamic tract to the thalamus and the cerebral cortex. Activation of the cerebral cortex in healthy subjects can be recorded and analyzed non-invasively using electroencephalographic recordings of laser-evoked potentials (LEPs). LEPs have been studied in healthy subjects (Bromm and Treede, 1984; Arendt-Nielsen, 1990a,b; Tarkka and Treede, 1993) and in patients with various lesions of the peripheral or central nervous system (see Bromm and Lorenz, 1998 for review). Thus, LEPs are a valuable tool to study the nociceptive pathways in humans and to document objective and quantitative correlates of lesions of these pathways.

Brief laser-induced heat pulses can elicit LEPs, since the cutaneous terminals of primary nociceptive neurons posses a very fast transduction mechanism for noxious heat (Treede et al., 1995). A likely candidate for this fast mechanism is the heat-activated inward current I_{heat} which has been investigated by the patch–clamp technique using the somata of dorsal root ganglion (DRG) neurons in vitro as models for their own peripheral nociceptive terminals. Noxious heat rapidly evokes I_{heat} in a subpopulation of small DRG neurons by opening of non-selective cation channels (Cesare and McNaughton, 1996; Kirschstein et al., 1997, 1999; Kress and Guenther, 1999; Vyklický et al., 1999;

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0304-3959/02/$20.00
PII: S0304-3959(02)00071-4

Nagy and Rang, 1999; Liu and Simon, 2000; Marín-Burgin et al., 2000). All of these studies have used stimulation with heated extracellular solution, which mimics heat stimulation by contact thermodes.

In contrast, many in vivo studies have used radiant heat stimuli, which avoid mechanical activation of nerve terminals, e.g. in order to map heat receptive fields of primary nociceptive afferents (Treede et al., 1990), to quantify the heat responses of convergent spinal neurons (Handwerker et al., 1975), to measure nociceptive behavior in animal models of neuropathic pain (Hargreaves et al., 1988), or to assess heat pain in areas of mechanical hyperalgesia (Ali et al., 1996). Laser radiant heat offers the additional advantage of generating very fast temperature increases at the skin surface (e.g. within 3 ms with an amplitude depending on the applied energy; Spiegel et al., 2000). Laser radiant heat stimuli have been used to investigate the fast heat transduction mechanism in correlative studies of action potential discharges in primary nociceptive afferents in animals as well as LEPs and psychophysical responses in humans (Meyer and Campbell, 1981; Treede et al., 1994). However, no such correlative studies have been done for heat-evoked currents in dissociated DRG neurons and human psychophysics.

The aim of this study was to compare I_{heat} with human psychophysics and LEPs using the same fast, contactless heat stimulus. We thus investigated whether I_{heat} can be elicited in vitro by stimulating the somata of DRG neurons of rats directly with infrared laser pulses and whether comparable laser stimuli elicit subjective pain sensations from the skin as well as objective LEPs in humans. For this purpose, we used a diode laser stimulator generating infrared radiation of a wavelength of 980 nm that exhibits relatively little absorption in water, because the laser radiation had to pass through a layer of skin or extracellular solution of varying thickness, before reaching the nociceptive nerve endings in the skin or the nociceptive DRG neurons in the in vitro recording setup.

Some of the findings have been reported in abstract form (Nemenov and Mikkelsen, 1999; Greffrath et al., 2001b).

2. Materials and methods

2.1. Preparation of acutely dissociated DRG neurons

Acutely dissociated DRG neurons were obtained as previously described (Kirschstein et al., 1997, 1999). Briefly, adult Sprague–Dawley rats (250–315 g) of either sex were deeply anesthetized with diethylether (Merck, Darmstadt, Germany) and rapidly decapitated. The spine was transferred into chilled F12-Dulbecco's modified Eagle's medium (Sigma, Deisenhofen, Germany; adjusted to pH 7.4 by NaOH) containing 30 mM $NaHCO_3$ (Merck), 100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin (Sigma), and equilibrated with 95% O_2/5% CO_2 throughout the whole preparation and dissociation procedure. DRGs were quickly dissected and dissociated at 37°C using collagenase CLS II (5–10 mg ml^{-1}, 40–50 min; Biochrome, Berlin, Germany) and trypsine (0.2–1 mg ml^{-1}, 10–12 min; Sigma). Neurons were triturated with fire polished Pasteur pipettes and plated on microscope cover glasses glued to the bottom of 35-mm diameter culture dishes. These dishes, which also served as recording chambers, were stored for 3–12 h in a humidified 5% CO_2 atmosphere at 37°C before being used for electrophysiological recordings.

2.2. Electrophysiological recordings in vitro

For whole-cell patch-clamp experiments, dishes were filled with 2 ml of extracellular solution (about 1 mm above cell level) containing (in mM) 145 NaCl, 2.5 KCl, 10 HEPES, 10 glucose, 1.5 $CaCl_2$ and 1.2 MgCl (pH adjusted to 7.4). Patch pipettes were fabricated from borosilicate glass capillaries (Hilgenberg, Malsfeld, Germany), fire polished and filled with a solution containing (in mM) 160 KCl, 10 HEPES and 8.13 EGTA adjusted to pH 7.2 by KOH ($R_{Tip} = 2.8 \pm 0.24$ MΩ; range 2–6 MΩ). Recordings were performed using an Axopatch 200A amplifier (Axon Instruments, Foster City, USA; four pole lowpass Bessel filter at 2 kHz) and pClamp8 Software (Axon Instruments; sampling rate: 10 kHz) in voltage-clamp (at −80 mV) and current-clamp mode (held at about −60 mV). Only round or oval DRG neurons without any processes and with a major diameter up to 30 μm (measured by a calibrated microscope eyepiece) were selected for recording, since in previous studies I_{heat} was only observed in small neurons (Kirschstein et al., 1997, 1999). After establishing the gigaseal and breaking through the cell membrane by gentle suction, membrane capacity and resting membrane potential were measured for each investigated neuron. Cells without the capability to generate action potentials elicited by short (40 ms) depolarizing current pulses where excluded from further investigation. In some experiments, capsaicin (1–10 μM; Sigma) was applied with a puffing system previously described (Kirschstein et al., 1997, 1999). Bath temperature was measured with an infrared temperature scanner (DT-1000; Exergen Corp., Newton, USA) before and after the series of laser stimuli in each experiment.

2.3. Diode laser stimulation

A personal computer controlled laser platform based on six GaInAs/GaAs laser diodes (980 nm wavelength) yielding up to 15 W output power into a flexible glass fiber core of 0.36 mm diameter was used for thermal stimulation in vitro and in vivo. One of the laser diodes was used for pumping a Nd:IAD laser (503 nm wavelength after frequency doubling, i.e. in the visible range) indicating the beam size of the irradiated spot. The fiber was mounted above: (1) DRG neurons in the bath; or (2) the fingers of human volunteers using a micromanipulator. The diode laser stimulator did not induce any significant noise neither when located near the electro-

encephalogram (EEG) recording setup nor near the electrophysiological setup in vitro. The beam diameter for induction of I_{heat} was adjusted to 1.2 mm at the bottom of the dish using a calibrated microscope eyepiece and to about 0.6 mm for eliciting pain sensations in humans using a stereo microscope. Stimulus intensity was changed by varying the laser power (2.8–11 W) and/or stimulus duration (4–400 ms). The interstimulus interval following the appearance of I_{heat} was at least 44 s.

To record the time course of the heat stimuli generated by laser pulses, open patch pipettes (R_{Tip} = 1.0–6.3 MΩ) were used in place of the DRG neurons as described previously (Cesare and McNaughton, 1996; Schwarz et al., 2000). To measure the peak temperatures with a miniature thermocouple (IT-1E, tip diameter about 230 μm; Physitemp, Clifton, USA) was impossible, as the thermal capacity of the probe was too high to follow the fast temperature changes (cf. Schwarz et al., 2000). Furthermore, the probe itself absorbs more energy than the DRG neurons due to its opaque structure and larger size.

2.4. Experimental protocol in vivo

Pain thresholds in healthy human volunteers (n = 9; one female, eight males, age 20–54 years) were determined by applying laser stimuli to the second or third finger using a staircase regime. All volunteers gave informed consent and the experiments were approved by the local ethics committee. Five subjects were stimulated at the dorsal side near the nail (hairy skin), six were stimulated at the fingertip (glabrous skin). Laser pulses of increasing power were applied at different constant stimulus durations (range: 50–300 ms). Pain thresholds were determined as the minimum laser power inducing a painful pinprick-like sensation at a given stimulus duration.

A three-channel EEG (electrodes: Fz, Cz, Pz) was recorded in a single human subject (male, 54 years) with Ag–AgCl electrodes (impedance <5 kHz) using ipsilateral ear reference with a bandpass of 0.1–500 Hz (sampling rate: 1 kHz). An electro-oculogram (EOG) was also recorded to identify artefacts induced by eye movements. The pulse duration of laser stimuli was 80 ms at an output power of 5.4 W and an interstimulus interval of 6 s. The site of stimulation in the hairy skin of the left forefinger was slightly changed following each stimulus in order to avoid nociceptor suppression and tachyphylaxis of I_{heat} (Meyer et al., 1994; Schwarz et al., 2000). Twenty single sweeps were averaged time-locked to the onset of stimulation. The averages were filtered offline with a lowpass filter of 8 Hz.

2.5. Data analysis

All data are presented as mean ± standard error of the mean (SEM) except for the definition of the threshold of I_{heat}. Effects were statistically analyzed using Student's t-test for paired and unpaired data. $P < 0.05$ was considered significant.

3. Results

3.1. Laser radiant heat pulses in an in vitro setup

To estimate the time course of the laser-induced heat stimuli, we applied laser pulses to open patch pipettes in place of the neurons. Due to the linear temperature dependence of the liquid junction potential, changes in holding currents are proportional to changes in temperature at the tip of the pipette (-0.14 ± 0.01°C pA^{-1}, according to Schwarz et al., 2000). The intensity of the laser pulses was changed by varying the stimulus duration (4–400 ms, Fig. 1A) and/or the power of the laser (2.8–11 W, Fig. 1B). These data indicate that the temperature stimuli induced by the diode laser pulses were ramp shaped. The peak latency was proportional to the stimulus duration (r = 0.999) and the slope of the temperature ramp was proportional to the applied power ($r = -0.961$, n = 27 measurements with the pipette used in Fig. 1). Fig. 1C demonstrates the temperature calibration for one pipette; based on the mean data from three pipettes, the temperature change induced by the maximum stimulus intensity (11 W, 400 ms) was estimated as 27 ± 2.3°C. The recovery of open pipette currents reflected the time course of passive cooling ($t_{1/2}$ = 52.9 ± 2.0 ms; n = 41 measurements in three pipettes).

These measurements also indicate the size of potential non-specific heat-induced effects due to direct laser heating of the recording pipette. In DRG neurons, only inward currents exceeding the mean + 3SD of the peak currents induced in open patch pipettes were considered to represent a specific I_{heat}. Depending on stimulus intensity, this criterion value varied between −40 and −430 pA (Figs. 2 and 3, dotted lines).

3.2. Cellular responses to laser-induced heat pulses in vitro

Thirteen small DRG neurons (diameter 27.3 ± 0.6 μm) were stimulated with the diode laser stimulator. These neurons had a resting membrane potential of −59.7 ± 1.7 mV and a membrane capacitance of 25.0 ± 1.2 pF. Depolarization by constant current injection elicited action potentials (amplitude baseline to peak 120 ± 2.5 mV) in every neuron. Bath temperature was not changed by the series of laser stimuli (mean temperature before, 26.1 ± 0.2°C; after stimulation, 26.1 ± 0.3°C; n.s.).

Laser stimuli changed the holding currents in all 13 neurons tested. In seven neurons, these changes in holding current were within the 3SD range of open pipettes even at the maximum stimulus intensity (dotted lines in Figs. 2 and 3). Thus, these neurons gave no evidence for a specific I_{heat} and were considered heat insensitive. An example of one of these neurons is shown in Fig. 2A. In contrast, six of the neurons responded with inward currents that clearly exceeded the range explained by non-specific heat effects in the recording setup and, therefore, were considered heat sensitive. Fig. 2B shows an example of one of those

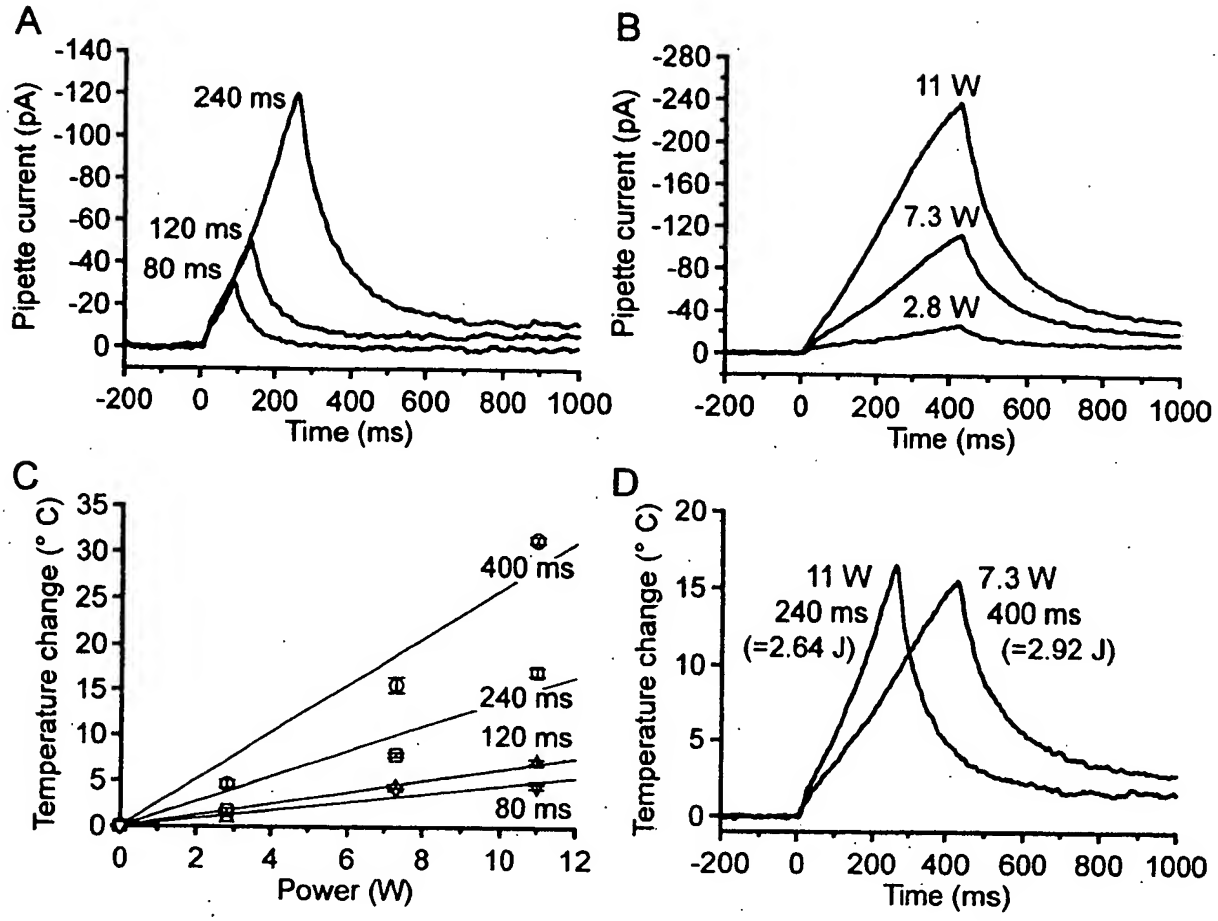

Fig. 1. Diode laser pulses induce ramp-shaped heat stimuli. Time courses of diode laser induced temperature changes were recorded with open patch pipettes. Each trace was averaged from two single measurements, filtered with an eight-pole Bessel filter (50 Hz) and a notch filter (center frequency, 50 Hz; width, 10 Hz). (A) Peak temperature was always reached at the end of the laser pulse, and was a function of pulse duration (power: 11 W). (B) Peak temperature was also a function of laser power (duration: 400 ms). (C) Calibration diagram for one pipette (mean ± SEM), based on the average temperature coefficient of -0.14 ± 0.01°C pA^{-1} determined in a previous study (Schwarz et al., 2000). For any given pulse duration, peak temperature was proportional to the applied laser power (O 400 ms; □ 240 ms; Δ 120 ms; ∇ 80 ms). Note that either 11 W for 240 ms or 7.3 W for 400 ms led to nearly the same peak temperature. (D) By simultaneously changing power and duration of the laser pulse, the same peak temperature was achieved with different slopes.

neurons. As shown in Table 1, other properties of heat-sensitive and -insensitive neurons did not differ significantly. All three heat-sensitive neurons tested were also sensitive to capsaicin, but so were four of five heat-insensitive neurons.

3.3. Kinetics of I_{heat} elicited by laser stimulation

Fig. 2 compares the time course of the effects of laser stimulation on holding currents in heat-insensitive and -sensitive DRG neurons. In a typical heat-insensitive neuron (Fig. 2A), the holding current rose linearly throughout the stimulus duration of 400 ms. The peak current was proportional to stimulus intensity, resembling the open pipette recordings in Fig. 1. Similar effects were seen for lower stimulus intensities in the heat-sensitive neuron (Fig. 2B), but the amplitude of its inward current suddenly reached about −3 nA at the first 11 W stimulus, indicating the appearance of a specific I_{heat}. In Fig. 2C, the responses of the two neurons to the first 11 W stimulus were superimposed. The holding currents in both neurons started to change linearly at stimulus onset and reflected the time course of the temperature ramp applied. In the heat-sensitive neuron, there was a delayed change in slope, which indicates the onset of the specific I_{heat} (cf. Cesare and McNaughton, 1996; Vyklický et al., 1999). The mean onset latency of I_{heat} for stimuli of 400 ms duration was 276 ± 20 ms ($n = 5$). The different kinetics of the non-specific and specific neuronal responses to laser stimulation can also be seen in the averaged responses of heat-insensitive neurons (Fig. 2D) and heat-sensitive neurons (Fig. 2E). Due to the non-linear time course of I_{heat}, the mean currents in heat-sensitive and -insensitive neurons were significantly different from 340 ms after stimulus onset to 50 ms after stimulus offset (open circles in Fig. 2E).

Once activated by laser stimuli, I_{heat} rapidly increased

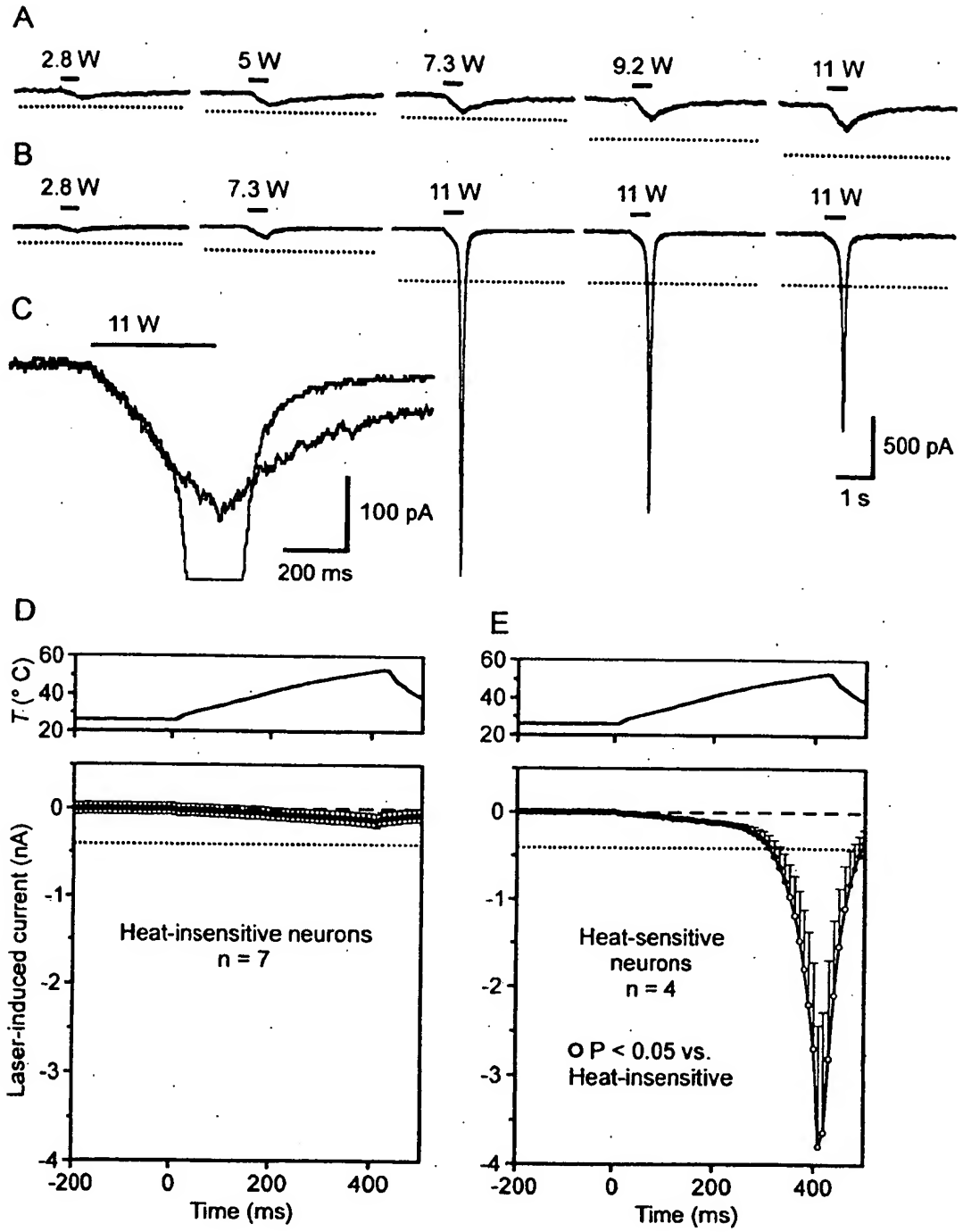

Fig. 2. Kinetics of heat-induced inward currents (I_{heat}) in DRG neurons. (A) In a typical heat-insensitive neuron (25 μm diameter, initial I_{hold} = +156 pA), laser stimuli up to 11 W and 400 ms duration induced small changes of the holding current that did not exceed the range of responses seen in the open pipette measurements (mean + 3SD, dotted lines). (B) In a typical heat-sensitive neuron (27.5 μm diameter, initial I_{hold} = −84 pA), similar effects were seen with stimuli of lower intensity but this neuron exhibited a large inward current (I_{heat}) when tested with 11 W for 400 ms. Induction of I_{heat} was reproducible but the peak current decreased, when the neuron was stimulated repetitively. (C) Magnification of the currents induced in both neurons by 11 W for 400 ms (filtered using a Gaussian lowpass filter at 100 Hz). Both inward currents initially increased linearly with the laser-induced linear rise in temperature. At 265 ms after stimulus onset, a large inward current was activated in the heat-sensitive neuron. (D) Population responses of seven heat-insensitive and (E) four heat-sensitive neurons stimulated with 11 W for 400 ms. Heat-insensitive neurons revealed a linear change in holding current throughout the stimulus; inward currents in heat-sensitive neurons were similar during the initial stimulation phase but increased significantly after reaching the threshold temperature of I_{heat} (open circles indicate $P < 0.05$ vs. heat-insensitive neurons, Student's unpaired t-test). Dashed lines mark a current of 0 nA, dotted lines mean + 3SD of open pipette measurements. Temperature (T) was estimated as in Fig. 1.

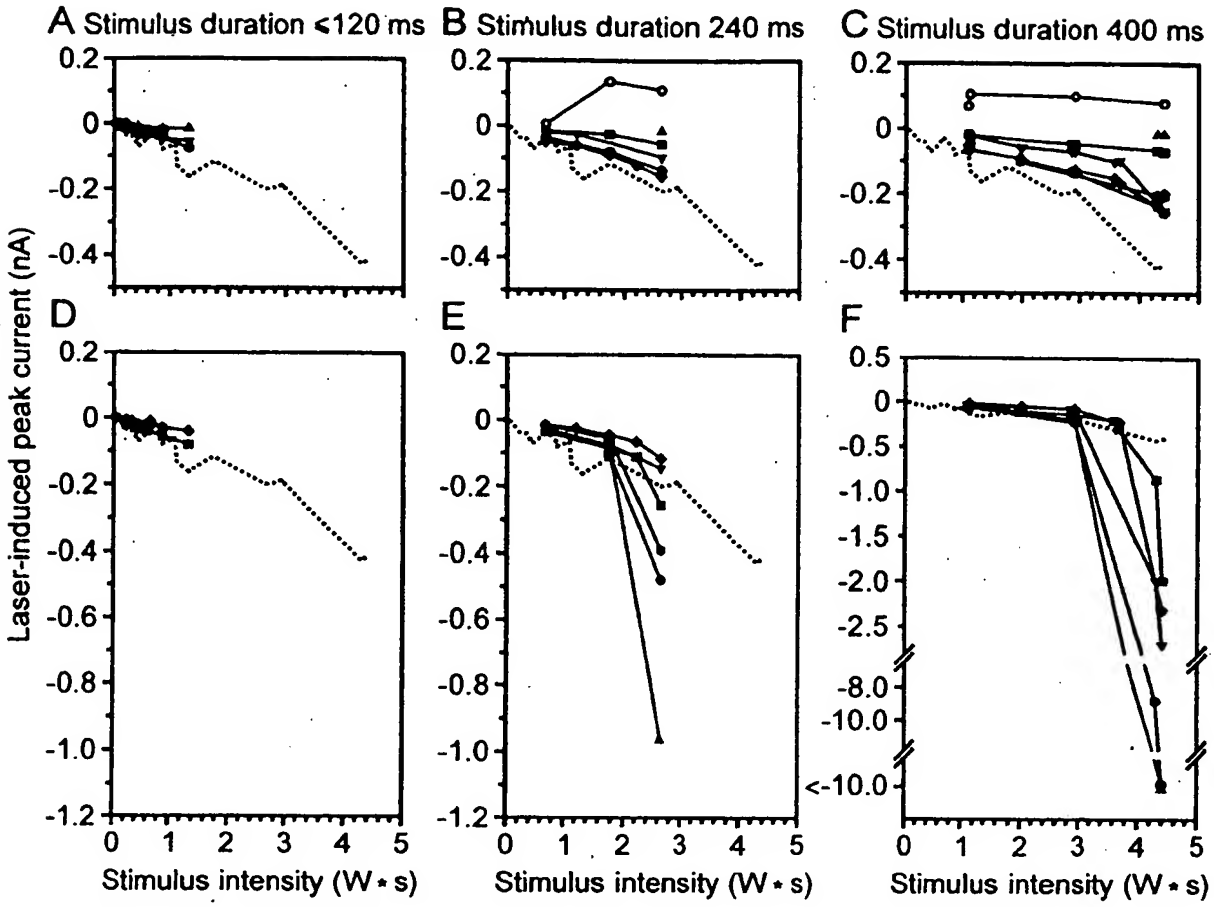

Fig. 3. Diode laser pulses activate I_{heat} in a subset of small DRG neurons. Laser stimuli of increasing power (2.8, 5, 7.3, 9.2 and 11 W) induced small changes in holding currents in seven small DRG neurons (A–C). The amplitude of these currents changed *linearly* with increasing stimulus intensity regardless of stimulus duration. As currents did not reach the threshold for a specific I_{heat} (mean + 3SD of the effect measured with patch pipettes alone; indicated as dotted line) these neurons were considered heat insensitive. In another subset of six small DRG neurons (D–F) inward currents increased *non-linearly* with stimulus intensity, clearly exceeding the range explained by non-specific heat effects (dotted line) when stimulated with 11 W for 240 ms (2.6 W s) or with 11 W for 400 ms (4.4 W s).

with time to reach 50% of the maximum ($t_{1/2}$) of 96.5 ± 5.9 ms ($n = 4$). The peak of I_{heat} approximately coincided with the end of the laser pulse (e.g. peak latency 415.3 ± 4.2 ms for 400 ms duration), indicating that I_{heat} started to deactivate with the onset of passive cooling. The $t_{1/2}$ to decrease by 50% was 27.7 ± 1.5 ms ($n = 4$), i.e. deactivation was faster than activation ($P < 0.005$, Student's paired t-test) and significantly faster than the mean temperature decay as estimated from the three open patch pipettes (54.8 ± 5.4 ms; $P < 0.01$ vs. heat-sensitive DRG neurons). In contrast, recovery in heat-insensitive neurons was broadly distributed (171.9 ± 55.3 ms, $n = 7$; range: 35.2–447.15 ms) and did not differ significantly from the mean temperature decay.

3.4. Rate dependence of the induction of I_{heat}

A linear increase of the amplitudes of heat-induced changes of holding currents over the whole range of stimulus intensities applied was seen in heat-insensitive neurons regardless of stimulus duration (Fig. 3, top traces), which was similar to currents in open pipettes. In contrast, currents in DRG neurons classified as heat-sensitive increased non-linearly with stimulus intensity (Fig. 3, bottom), with a significant, markedly rising I_{heat} appearing when neurons were stimulated with a power of 11 W for ≥240 ms.

The mean I_{heat} was significantly larger than the non-specific effects in heat-insensitive neurons at 11 W × 240 ms and at 11 W × 400 ms ($P < 0.05$). This response pattern is remarkable, because no I_{heat} was seen at an intermediate intensity (7.3 W × 400 ms) that led to the same peak temperature (about 43°C, Fig. 1D) as the 11 W × 240 ms stimulus. These data show that induction of a specific I_{heat} depended on both, the peak temperature induced by the laser pulse, and the rate of temperature change.

In all heat-sensitive neurons, the first suprathreshold I_{heat} was in response to a stimulus of 11 W, which increased temperature at a rate of about 70°C s^{-1}. Since the supra-

Table 1
Properties of heat-sensitive and heat-insensitive small neurons*

	Heat-sensitive neurons		Heat-insensitive neurons
Number of neurons	6		7
AP shoulder present	6 of 6 neurons		7 of 7 neurons
Capsaicin responders	3 of 3 neurons		4 of 5 neurons
Diameter (μm)	27.1 ± 0.7 (6)	n.s.	27.5 ± 1.0 (7)
Capacitance (pF)	26.3 ± 1.2 (6)	n.s.	23.9 ± 1.9 (7)
R_{Tip} (MΩ)	2.6 ± 0.2 (6)	n.s.	3.1 ± 0.5 (7)
Membrane potential (mV)			
Before heat	−61.7 ± 1.0 (6)	n.s.	−61.6 ± 1.9 (7)
After heat	−61.8 ± 1.8 (4)	n.s.	−65.3 ± 3.9 (7)
AP amplitude (mV)			
Before heat	118 ± 2.5 (6)	n.s.	122 ± 4.0 (7)
After heat	119 ± 2.0 (4)	n.s.	124 ± 6.7 (7)
AP threshold (nA)			
Before heat	−2.17 ± 0.23 (6)	n.s.	−2.17 ± 0.31 (7)
After heat	−0.95 ± 0.08 (4)	n.s.	−2.41 ± 0.65 (7)
Maximum I_{heat} (nA)	−4.6 ± 1.6 (6)	*	−0.13 ± 0.05 (7)
I_{caps} (nA)	−1.8 ± 0.09 (3)	n.s.	−2.8 ± 1.1 (4)
Time to peak I_{caps} (s)	10.2 ± 4.2 (3)	n.s.	11.1 ± 2.1 (4)

* Mean values ± SEM (number of neurons tested). AP, action potential; R_{Tip}, tip resistance of the patch pipette; I_{heat}, heat-evoked current (in two neurons, I_{heat} caused amplifier overload for the first maximal stimulus; these values were replaced by the maximal reading of 10 nA); I_{caps}, capsaicin-evoked current; unpaired t-test: n.s., not significant, * = $P < 0.05$.

threshold stimulus durations differed between neurons (on average 293 ± 31 ms), the mean threshold for the induction of I_{heat} was 3.2 ± 0.3 J. According to Fig. 2C, the temperature threshold of I_{heat} was estimated from its onset latency (change in slope at 222 ± 18 ms and −145 ± 10 pA), which yielded a value of 42 ± 1°C.

3.5. Neuronal response properties following heat stimulation

Four heat-sensitive neurons were stimulated repetitively (three to six pulses). In all neurons tested, each repetition of an initially suprathreshold stimulus again resulted in the induction of a specific I_{heat}. The amplitude of I_{heat}, however, decreased when a heat-sensitive neuron was stimulated repetitively, e.g. in the neuron shown in Fig. 2B I_{heat} decreased by 17.0% at the second, by 41.8% at third and by 39.4% at the fourth 11 W stimulus. Similar observations were made in the other neurons.

Excitability by electrical pulses was tested after the series of laser stimuli in 11 neurons (four heat-sensitive and seven heat-insensitive). Depolarizing current pulses still elicited action potentials (amplitude 119 ± 2.0 mV in heat-sensitive vs. 124 ± 6.7 ms in heat-insensitive neurons, n.s.) indicating that neurons were not damaged by laser stimuli. The inward current necessary to induce action potentials decreased after laser stimulation in heat-sensitive neurons (pre, −2.3 ± 0.27 nA; post, −0.95 ± 0.08 nA; $P < 0.05$) suggesting an increase of excitability in heat-sensitive neurons after laser stimulation. No such sensitization was observed in heat-insensitive neurons (pre, −2.17 ± 0.31 nA; post, −2.41 ± 0.65 nA; n.s.).

3.6. Psychophysics and LEPs in humans

Stimulation of human skin with the diode laser induced pain sensations in all nine human volunteers tested. Five volunteers were tested in hairy skin, six in glabrous skin (Table 2). Pain thresholds of hairy and glabrous skin did not differ significantly at any of the pulse durations tested

Table 2
Thresholds for heat-evoked currents and pain sensation

	DRG neurons ($n = 6$)	Glabrous skin ($n = 6$)	Hairy skin ($n = 5$)
Threshold (J)*	3.2 ± 0.3	0.76 ± 0.05	0.64 ± 0.10
Diameter (mm)	1.2	0.6	0.6
Area (mm^2)	1.13	0.28	0.28
Threshold (J mm^{-2})	2.8 ± 0.3	2.7 ± 0.2	2.3 ± 0.3

* Because of the rate dependence of thresholds, only values obtained with stimulus durations of 200 ms and above were averaged.

(50–300 ms). When painful laser stimuli of near threshold intensity (5.4 W, 80 ms) were applied repetitively in one subject, it was possible to obtain reliable LEPs in the vertex leads. The peak latencies of the first negativity (−0.8 μV at 285 ms) and positivity (3.4 μV at 490 ms) correspond to a typical response to A-fiber nociceptor activation, the small second positivity (2.9 μV at 1000 ms) may be due to C-fiber nociceptor activation.

For stimulus durations ≥200 ms, the mean pain threshold pooled across hairy and glabrous skin was 0.7 ± 0.05 J and the mean threshold for the induction of I_{heat} was 3.2 ± 0.3 J. This difference, however, is explained by the difference in the size of the irradiated area (Table 2). When threshold energies were normalized to the irradiated areas, the mean human pain threshold (energy density 2.5 ± 0.2 J mm^{-2}, $n = 11$) and the mean threshold for the induction of I_{heat} (energy density of 2.8 ± 0.3 J mm^{-2}, $n = 6$) were not significantly different, indicating that I_{heat} was induced by diode laser stimuli that were painful when applied to the human skin.

4. Discussion

This study has shown that diode laser stimuli that are painful when applied to the hairy or glabrous skin of the human hand also elicit inward currents (I_{heat}) in a subset of heat-sensitive rat DRG neurons. The onset latency of I_{heat} induced by radiant heat stimuli indicated a threshold temperature near 42°C; this threshold value and the non-linear temperature dependence of I_{heat} suggest that it may be mediated by the heat-sensitive vanilloid receptor channel VR1 (Caterina et al., 1997). Although similarity in appearance does not prove identity in mechanisms, this hypothesis is further supported by the observation that all heat-sensitive neurons tested were excited by the vanilloid receptor agonist capsaicin. Two previous studies have tested the effects of laser radiation on cultured nodose ganglion and DRG neurons (Miura and Kawatani, 1996; Jimbo et al., 1998). Due to the low power of the lasers used (16–150 mW), these stimuli were insufficient to elicit any thermal effects. In another study, cultured trigeminal ganglion neurons were heated indirectly by diode laser radiation (970 nm wavelength, 500 mW) that was directed to the blackened tip of an optical fiber near the cell (Baumann and Martenson, 1994); some of the neurons responded with trains of action potentials to heat stimuli of 45°C. In the present study, we used nearly the same wavelength for direct laser irradiation of DRG neurons and recorded inward currents in voltage clamp mode that exceeded the action potential threshold determined with electrical pulses.

4.1. Diode laser stimuli induce heat-evoked inward currents (I_{heat}) in nociceptive neurons

Due to the pronounced temperature dependence of all biological processes, rapid heating of a neuron by a laser pulse may also exert non-specific effects on its membrane potential (cf. Schwarz and Eikhof, 1987). To identify specific heat-evoked currents, we recorded the effects of the diode laser pulses on the recording setup in the absence of a neuron and used the 3SD range from the mean of such open pipette currents as a cutoff. In the subset of small DRG neurons that were thus classified as heat sensitive, the inward currents induced by the diode laser exhibited a non-linear temperature dependence. This non-linearity is reminiscent of heat-evoked currents elicited by ramp-shaped stimuli using superfusion of heated extracellular solution in cultured DRG neurons of neonatal rats (Cesare and McNaughton, 1996), acutely dissociated DRG neurons of adult rats (Vyklický et al., 1999; Nagy and Rang, 1999), and acutely dissociated rat trigeminal ganglion neurons (Liu and Simon, 2000). In the present data, this non-linearity was seen both within the response to an individual temperature ramp and across different stimulus intensities.

The heat-sensitive cation channel VR1 also exhibits a non-linear temperature dependence when expressed heterologously in *Xenopus* oocytes, human embryonic kidney cells (HEK293) or chinese hamster ovary cells (Caterina et al., 1997; Tominaga et al., 1998; Hayes et al., 2000). Since VR1 is gated by binding the irritant substance capsaicin, a fast I_{heat} is usually observed in DRG neurons that are also sensitive to capsaicin (Kirschstein et al., 1997, 1999; Vyklický et al., 1999; Nagy and Rang, 1999). Slower heat responses with a higher threshold in capsaicin-insensitive DRG neurons may be related to another heat-sensitive cation channel VRL1 (Caterina et al., 1999; Nagy and Rang, 1999). In the present study, I_{heat} was observed in capsaicin-sensitive neurons, exclusively. The percentage of heat-sensitive neurons (46%) was smaller than reported previously. Since most of the heat-insensitive neurons were capsaicin-sensitive, we cannot exclude that some of them might have responded with a specific I_{heat} to diode laser pulses of higher intensity.

In the subset of small DRG neurons that were classified as heat-insensitive, the currents induced by the diode laser exhibited a linear temperature dependence similar to the one reported for non-nociceptive large DRG neurons, sympathetic ganglion neurons, and heat-insensitive small DRG neurons (Cesare and McNaughton, 1996; Kirschstein et al., 1997, 1999; Vyklický et al., 1999; Nagy and Rang, 1999; Greffrath et al., 2001a). In the present data, this non-specific change in holding current amounted to less than 3% of I_{heat} in the heat-sensitive neurons. This percentage is similar to values reported previously, which ranged from 5 to 22% (cf. Greffrath et al., 2001a). HEK293 cells and oocytes without VR1-transfection also exhibit similarly small inward currents with a linear temperature dependence (Caterina et al., 1997; Tominaga et al., 1998; Hayes et al., 2000). It is not presently known to what extent heat-sensitive signal transduction pathways other than VR1 and/or the general temperature dependence of membrane conductance that contribute to the resting potential are involved in these small non-specific heat responses.

4.2. Properties of the laser-induced I_{heat}

The laser-induced I_{heat} activated with a sharp increase in inward current (cf. Cesare and McNaughton, 1996; Caterina et al., 1997; Tominaga et al., 1998; Vyklický et al., 1999). Deactivation of I_{heat} was even faster than its activation and occurred almost simultaneously with the onset of passive cooling. This rapid deactivation of I_{heat} has been noted before in nociceptive DRG neurons and in VR1-transfected cells (Cesare and McNaughton, 1996; Tominaga et al., 1998; Schwarz et al., 2000). Likewise, heat-evoked action potential discharges recorded in vivo stop immediately when the temperature starts to decrease, even if it remains above the initial threshold (Meyer and Campbell, 1981; Tillman et al., 1995).

When tested repetitively, the laser-induced I_{heat} tended to decrease; this tachyphylaxis has been described previously for I_{heat} induced by superfusion with heated solutions (Schwarz et al., 2000). Action potential discharges in vivo and pain sensations in humans show a similar trend to decrease with repetitive short heat stimuli (Adriaensen et al., 1984; Treede, 1995). Repetitive heat stimuli are also known to cause sensitization to heat and its psychophysical correlate, primary hyperalgesia to heat (Meyer et al., 1994). So far, sensitization of I_{heat} by preceding heat stimuli has not been observed (Cesare and McNaughton, 1996; Kirschstein et al., 1997, 1999; Vyklický et al., 1999; Nagy and Rang, 1999; Schwarz et al., 2000). This discrepancy suggests that sensitization by heat to subsequent heat stimuli may only occur at the transformation process into action potentials instead of the initial step of sensory transduction.

In the present study, we observed a significant drop in action potential threshold following a series of laser induced heat stimuli that was restricted to the population of heat-sensitive DRG neurons (cf. Jimbo et al., 1998). Since nociceptive DRG neurons express a specific set of sodium channels that are characterized by their resistance to blockade by tetrodotoxin, intracellular signal transduction pathways that specifically change the kinetics and/or expression of these channels are potential candidates to mediate primary hyperalgesia to heat. For the sensitizing effects of prostaglandins, such pathways have already been identified (Gold et al., 1996, 1998).

4.3. Pain sensation and absorption of diode laser radiation in vivo and in vitro

The diode laser used in the present study allows direct heating of nerve terminals through extracellular solutions and skin. Its infrared radiation (wavelength 980 nm) has a long extinction length in water and tissue of 3800 μm (Jacques, 1996; Nemenov et al., 1996). This extinction length is comparable to that of the visible argon laser light, but not other infrared lasers that have been used in human pain research (cf. Svensson et al., 1991). As a consequence, the diode laser pulses could penetrate the stratum corneum overlying the nociceptive nerve terminals in hairy skin (about 20 μm thick) or glabrous skin (several hundreds μm thick) as well as the overlying extracellular solution covering the dissociated DRG neurons (about 1000 μm thick). Thus, the low absorption of diode laser pulses may explain why pain thresholds in hairy and glabrous skin as well as the threshold to induce I_{heat} were similar. For carbon dioxide laser pulses, pain thresholds in hairy skin were about 50% lower than in glabrous skin (Pertovaara et al., 1988), probably because infrared radiation of that wavelength (10 600 nm) has an extinction length of only 10 μm.

Like for other types of laser-induced heat pulses, it was possible to detect an evoked potential in the EEG following diode laser stimuli (cf. Gülsoy et al., 2001). The prominent positive peak of this LEP (peak latency: 490 ms) corresponded to the so-called late component of the LEP mediated via Aδ-fibers (Kenton et al., 1980; Bromm and Treede, 1987; Arendt-Nielsen 1990a; Kakigi et al., 1991). The second response at a latency of about 1000 ms may be an ultralate potential due to C-fiber activation (Bromm et al., 1983; Arendt-Nielsen 1990b; Bragard et al., 1996). Conduction velocities and thresholds of these LEP components suggest that they are mediated by type II A-fiber mechano-heat nociceptors and by C-fiber mechano-heat nociceptors, respectively (Treede et al., 1994). These two classes of afferents are thought to express the putative heat transducer channel VR1, as shown, e.g. by the finding that LEP could be blocked by long-term treatment with capsaicin cream (Beydoun et al., 1996).

5. Conclusions

Infrared diode laser pulses that are painful for human subjects, activate an inward current in primary nociceptive neurons in vitro that shares many properties with I_{heat} that has been studied with application of heated solutions. As I_{heat} is likely mediated by opening of the vanilloid receptor channel VR1, brief heat stimuli generated by infrared lasers offer the opportunity to study the functional integrity of VR1-expressing nerve fibers in humans. The LEP data in humans showed a latency that corresponded to activation of myelinated afferents. This observation suggests that the diode laser pulses activated type II A-fiber mechano-heat nociceptors, a class of polymodal nociceptive afferents that are thought to express the fast I_{heat} and VR1. Diode lasers are adaequate as non-contact heat stimulators for human pain research, which rapidly activate capsaicin-sensitive nociceptive afferents relatively independent of their depth of termination within the skin.

Acknowledgements

The authors thank V.G. Zaitcev for developing the laser control software, W. Magerl for statistical support, G. Günther and G. Schatt for technical assistance and J.

Mikkelsen and J.Nielsen for help with the psychophysical experiments. This study was supported by the Deutsche Forschungsgemeinschaft Grant Tr236/11-1 and 11-2.

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ATTACHMENT 2

Centenary of I.P.Pavlov Medical State University

1897-1997

SEMICONDUCTOR AND SOLID STATE LASERS IN MEDICINE 97

International Workshop
May 24-26 1997
St.Petersburg Russia
Organized by I.P.Pavlov Medical State University
Sponsored by
St.Petersburg Social Foundation of Culture and Education & Firm "Soyuz"
Meeting Committees: N. N. Petrishchev, M. I. Nemenov, R.Chapman, A.J.Nevorotin, S.Plouznikov, L.A.Mikhailova

Saturday, May 24, 1997

09:00-09:30 Opening Ceremony

09:30-10:30 Opening Session:

Prospective of Semiconductor and Solid State Lasers in Medicine

Chairman: Nikolai N.Petrishchev,

Laser Medical Centre at Pavlov Medical University

0.1 *Semiconductor Lasers the Prospectives in Medicine*

R.Suris, M. Nemenov, Ioffe Physico-Technical Institute, Russia

15:30-16:30 Session III:

Laser Radiation and Somatic Sensitivity

Chairmen: Yuri D.Ignatov, Pavlov Medical University

Roxana Chapman, University College Hospital

3.1 *The Use of Argon Laser for Sensory Testing in Humans*

Lars Arendt-Nielsen, University of Aalborg, Denmark

3.2 *Skin Sensation and Laser Radiation as Universal Stimuli*

Mikhail I.Nemenov, Efim M.Tsirulnikov

Laser Medical Centre, Pavlov Medical State University

ATTACHMENT 3

Message-ID: <34C597EC.1EDC@ms.ire.rssi.ru>
Date: Wed, 21 Jan 1998 09:38:36 +0300
From: "Igor Samartsev" <sam@ms.ire.rssi.ru>
Organization: IRE-POLUS
X-Mailer: Mozilla 3.01 (Win95; I)
MIME-Version: 1.0
To: NEMENOV <nemenov@ldiode.ioffe.rssi.ru>
Subject: spec
Content-Type: multipart/mixed; boundary="------------651575DC66EF"
Lines: 3973
Content-Type: text/plain; charset=us-ascii
Content-Transfer-Encoding: 7bit
Content-Length: 292086
Status: RO

This is a multi-part message in MIME format.

--------------651575DC66EF
Content-Type: text/plain; charset=koi8-r
Content-Transfer-Encoding: 7bit

Dear Mechael I.

SPECIFICATION

MODEL	DL-5	DL-10	DL-40
Optical Characteristics:			
Nominal Output Power, W	5	10	40
Max.Output Power, W	7	15	60
Laser Wavelength, nm		970+-15	
Spectral Bandwidth, nm	10	10	15
Output Fiber Diameter	200	250	400
Output Beam NA	0.2	0.2	0.3
General Characteristics:			
Operating Temperature Range		10-50	
Power Requirements, V	12	12-15	24
Electrical Power Consumption	<20	<45	<180
Size, mm		168x243x67	

See also attached file.

Best Regardes
Igor

ATTACHMENT 4

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PROFORMA INVOICE

INVOICE NO: **IPG/PINV-80629**

DATE : 29.06. 1998

TO : Center for Sensory-Motor Interaction
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Shipped To: Center for Sensory-Motor Interaction
Fredrik Bajers Vej 7, D-3
DK-9220 Aalborg
Denmark

Buyer : Prof. Lars Arendt-Nielsen

This is supply within the European Community
Customer's VAT Ref. No. DK 32858910

PO NUMBER	PO DATE	SHIPPED VIA	F.O.B. POINT	TERMS
87965	28.04.1998	SIS	ExWorks Burbach	-

ITEM	DESCRIPTION	QTY	UNIT PRICE,	AMOUNT,
1	**Model DL-20,** **20W output power laser diode module** **incl. Green Lightning Option**	1	USD 13,400.00	USD 13,400.00
			SUBTOTAL	USD 13.4 00.00
			PACKAGING & AIR FREIGHT CHARGES & INSURANCE	-
			TOTAL DUE	**USD 13,4000.00**

Price Terms: Any other charges are not included.

This **Proforma Invoice** is for customs purposes only, not for payment.

Authorized by

IRE-POLUS Group

IPG Laser GmbH • Postfach 12 49 • D-57292 Burbach

Center for Sensory-Motor Imteraction
Fredrik Bajers Vej 7, D-3
DK-9220 Aalborg
Denmark

Attn.: Professor Lars Arendt-Nielsen
TEL: +45 96 35 88 30

IPG Laser GmbH

Siemensstraße 7
D - 57299 Burbach / Germany
Postfach 12 49
D - 57292 Burbach / Germany

Telephone + 49(0)2736 4420-0
Telefax + 49(0)2736 4420-25
E-Mail 101703.2430@ compuserve.com

Burbach, 29 June, 1997

PACKING LIST

ITEM	DESCRIPTION	QTY
1	**Model DL-20,** **20W output power laser diode module** **incl. Green Lightning Option** **(S/N : 05.566)**	1

Authorized by

IRE-POLUS Group

IPG Laser GmbH • Postfach 12 49 • D-57292 Burbach

SIS
Intern. Speditions-GmbH
Lotzenbachstraße 1

57290 Neunkirchen

Fax-Nr. 02735/789355

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IPGLaser@t-online.de

Burbach, 30. Juni 1998

Speditionsauftrag
Versand per Courier ☒ / Luftfracht ☐

Wir beauftragen Sie mit der Abholung und dem Versand von:

Verpackung	Markierung	Brutto-Gewicht	Abmessungen in cm
1 Karton	Adresse	10 kg	62 x 42 x 30 cm
Inhalt/Warenbezeichnung:			
1 Laserdioden-Modul Modell DL-20			

Empfänger: Center for Sensory-Motor Interaction
Frederik Bajers Vej 7, D-3
DK - 9220 Aalborg
Dänemark

USt-IdNr: DK 32858910

Attn.: Prof. Lars Arendt-Nielsen

Tel.: + 45 96 35 88 30

Frankatur:
☐ unfrei (ab Werk)
☐ fob Flughafen
☐ frei Bestimmungsflughafen
☒ frei Haus verzollt - ☐ incl. Zoll

Versicherung:
☒ durch SIS zu decken
Versicherungswert: DM 24.000,00

Wert für Zollzwecke: DM 24.000,00

Anlage:
☐ Ausfuhrerklärung Nr.
☐ Warenverkehrsbescheinigung EUR.1
☒ Proforma-Rechnung
☐ Handelsrechnung
☒ Lieferschein
☐

Wir erklären hiermit, daß es sich um kein Gefahrengut handelt.
Sofort nach erfolgtem Versand schicken Sie uns bitte eine AWB-Kopie.
Die Sendung wurde ohne äußerlich erkennbare Mängel übernommen.

IPG Laser GmbH

(Unterschrift des Abholers)

(Unterschrift des Versenders)

ATTACHMENT 5

Comments: Authenticated sender is <treede@pop.uni-mainz.de>
From: "R.-D. Treede" <treede@mail.Uni-Mainz.de>
To: Mikhail Nemenov <mn@smi.auc.dk>
Date: Mon, 2 Nov 1998 15:29:44 +0000
Subject: Re: (laser diodes for pain research
Reply-to: treede@mail.Uni-Mainz.de
Priority: normal
X-mailer: Pegasus Mail for Windows (v2.01)
Content-Type: text
X-UIDL: 29061f9afd9fc41516a8092f0175d802
Status: U
X-Mozilla-Status: 8013

Dear Dr. Nemenov,
this work of yours sounds very interesting. What is the wavelength of your laser diode radiation? Please do send your publications and curriculum vitae.
RDT

> Date: Mon, 02 Nov 1998 14:16:55 +0100
> From: Mikhail Nemenov <mn@smi.auc.dk>
> Organization: Aalborg university
> To: treede@mail.Uni-Mainz.de
> Subject: (laser diodes for pain research

> Dear Prof. Treede,
>
> I would to suggest you a new equipment and methods for skin pain
> research. I hope it will be interesting for you. The method and equipment is following:
> Laser diode set up with fibre delivery and methods which allow to
> evoke almost all monomodal skin sensations (incl. pin prick pain, warmth, itching, burning, tactile and etc.). It is possible to evoke very precisely threshold level of sensations and it is possible very precisely to change stimulus
> parameters. Irradiated spot can be have size up to 20-50 microns like
> tip of needle or smaller.
>
> Laser diode (LD) is not so dangerous as CO2, computer controlled
> and portable and cheaper.
> I think the difference from Tu laser is direct current modulation of
> laser diode, so controllability level of LD of course higher.
>
> So the level of sensations in deference which usually used is
> threshold. It was not supra threshold level, likes a standard for LEP pain research.
>
> And we calculated three dimensional temperature distribution compare
> you and Meyer.
>
> We used threshold level and we evoked sensations from area diameter
> up to 100 microns. Thus, it was possible to evoke threshold monomodal sensation from area related to single free nerve ending.
>
> Now I finish in Pain Lab Aalborg University as visiting researcher
> and I am looking for new contract.
>

> I can send you my last abstract and papers. But I need in your mail
> address.
>
> Yours sincerely,
> Michael Nemenov, Ph.D.
>
> Center for Sensory-Motor Interaction (SMI)
> Aalborg University
> Fredrik Bajers Vej 7, D2-111
> DK-9220 Aalborg
> Denmark
>
> Tel. +45 96 35 87 98
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Rolf-Detlef Treede
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Johannes Gutenberg University
Saarstr 21, D-55099 Mainz, Germany
phone +49-6131-395715, FAX +49-6131-395902

ATTACHMENT 6

JOHANNES GUTENBERG-UNIVERSITÄT MAINZ

Institut für Physiologie und Pathophysiologie
Fachbereich Medizin

universität mainz

D-55099 Mainz
Saarstr. 21
Telefon (06131) 39-25715
Telefax (06131) 39-25902
e-mail: treede@mail.uni-mainz.de

Immigration & Naturalization Service

16. August 2001

Re: Assistant Professor Dr. Mikhail I. Nemenov

Dear Sirs,

it is my pleasure to write a letter of recommendation for Dr. Mikhail Nemenov in support of his petition for an immigrant visa as an outstanding researcher with extraordinary ability.

Dr. Nemenov's outstanding achievements in optoelectronics and laser medicine have been recognized by the international scientific community as a result of his patented inventions, contributions at international conferences, and publications in leading peer-reviewed journals. Dr. Nemenov's findings have a number of important applications in the optoelectronics industry, as well as in biomedical research.

I got to know Dr. Nemenov in 1998, while he was a Visiting Scientist at the Sensory-Motor Interaction Center of Aalborg University in Denmark. Our colleagues in Aalborg as well as my own laboratory have a long standing record of research and development of stimulation devices for pain research using laser technology. Dr. Nemenov had been invited to Aalborg to test and verify the application of a novel laser diode stimulator for pain research in humans, where it is of use for characterizing damage to the nervous system and for clinical studies in analgesic drug development. Dr. Nemenov had previously developed this laser diode device in St. Petersburg, Russia. At the 1999 World Pain Congress in Vienna, I had the pleasure to meet Dr. Nemenov in person, where he presented his diode laser device and the very interesting scientific findings obtained with it.

Dr. Nemenov immediately impressed me with his sharp intellect and broad knowledge in laser physics, paired with his skills for interdisciplinary communication with neurobiologists. We decided to invite him to spend some time for research on the mechanisms of the activation of nociceptive neurons (the sensory cells that are specialized to detect tissue damaging and painful stimuli) in my laboratory.

In September 1999, Dr. Nemenov worked at my laboratory in Mainz, Germany. He arrived with a well prepared set of scientific questions that we were then able to address in a very intense time of experimental scientific work. Dr.Nemenov's scientific work was characterized by innovative ideas and outstanding speed. Scientific discussions with him were always held at the highest level. The results of this collaboration are important for studying basic mechanisms of pain as well as for preclinical studies of analgesic drug development. These joint findings have been submitted for publication to the top ranking journal in pain research.

In addition to his most recent work on the application of laser devices for studying the neurobiology of pain, Dr. Nemenov has a vast background in many areas of laser physics, optoelectronics, and laser medicine. In his native Russia, he graduated in physics from the most prestigious A.F. Ioffe Physical-Technical Institute in St. Petersburg (then Leningrad, USSR). He worked at several Physics and Medical departments of the Pavlov University in St. Petersburg, Russia, which is one of the leading research institutions in that country. Lateron, he was a Visiting Scientist at several renowned scientific institution in Europe.

As listed above, the curriculum vitae of Dr. Nemenov shows that he has contributed significantly to the scientific work at several centers of excellence in a number of countries. Dr. Nemenov has demonstrated his extraordinary scientific ability mostly within the field of optoelectronics, but more recently has also contributed significantly to biomedical research (particularly pain research). There is no question that Dr. Nemenov ranks among the academic elite of research professionals.

In summary, I believe that Dr. Nemenov's continued presence in the United States will benefit the American public and enhance the quality of research in your country, because of his advanced degree, his exceptional ability as an outstanding researcher, and his significant contributions to the field of applied optoelectronics and laser medicine, both within the United States and worldwide. Therefore, I fully support Dr. Nemenov's petition for an immigrant visa.

Sincerely,

Dr.med. Rolf-Detlef Treede
Professor of Neurophysiology

Encl. (Curriculum vitae)